

The Effect of Fibrin Structure on Fibrinolysis*

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Fibrin structure contributes to the regulation of the fibrinolytic rate. As the fibrin fiber size is decreased, the fibrinolytic rate also decreases. Fibrin structure was altered by either changing the ratio of thrombin to fibrinogen, *i.e.* altering the assembly rate or by adding a fibrin assembly inhibitor, iopamidol. Changes in the fibrinolytic rate were followed by measuring the time dependence of the decrease in the fiber mass/length ratio during fibrinolysis. A measure of the overall fibrinolytic rate was determined from the decrease in the mass/length ratio *versus* time. An 8-fold reduction in the fibrinolytic rate was seen on decreasing the mass/length ratio from 2.7×10^{12} daltons/cm to 0.5×10^{12} daltons/cm. It is shown that thin fibrin fibers have a decreased rate of conversion of plasminogen to plasmin by tissue plasminogen activator and that thin fibrin fibers are lysed more slowly than thick fibrin fibers.

The physiologic dissolution of an *in vivo* fibrin clot results from an interdependent process involving the interaction of tissue plasminogen activator (tPA)¹ and plasminogen in intimate contact on a fibrin surface (1). The adsorption of tPA and plasminogen on the fibrin fiber surface allows the necessary contact needed to affect optimal activation rates for plasmin production and spatial proximity of the fibrin substrate. Fibrin plays a dual role in this series of events. Not only does it enhance tPA activation, it also acts as substrate for plasmin.

The conversion of plasminogen to plasmin can occur by several different mechanisms, but it appears that the most important *in vivo* activator is tPA (2). tPA, $M_r \approx 70,000$, is present in plasma as a single-chain serine protease, but proteolytic cleavage of the Arg²⁷⁵-Ile²⁷⁶ bond in tPA by plasmin yields a disulfide-linked two-chain enzyme (3). The presence of fibrin greatly accelerates the activation of plasminogen by tPA and hence can exert a regulatory influence over plasminogen activation (4–6). Molecular details of the tPA-fibrin interaction are not completely understood. Structural features in the D-domain of fibrin contained in the peptide sequence A α ^{148–207} are crucial to tPA binding (6). Within this sequence, the lysine side chain at A α ¹⁵⁷, distal to the outer disulfide ring, has been implicated for the tPA binding (7). Voskuilen

et al. (7) postulate that fibrin formation exposes this latent tPA-binding site in the D-domain and thus facilitates plasminogen activation.

Plasmin, a disulfide-linked two-chain serine enzyme, is formed from the limited proteolysis of Arg⁵⁶⁰Val⁵⁶¹ bond in plasminogen (8). The heavy chain of plasmin contains the lysine-binding sites which are necessary for the binding of plasmin to fibrin. These sites are found in the so-called kringle regions (9). The light chain of plasmin contains the serine-active site. Several different forms of plasmin can be produced, depending on the presence of inhibitors. Under physiological conditions, Glu₁ plasmin predominates (10). In the absence of inhibitors, cleavage of the amino terminal portion of plasmin occurs between Arg⁶⁷-Met⁶⁸, Lys⁷⁶-Lys⁷⁷, or Lys⁷⁷-Val⁷⁸ to produce three similar forms of plasmin collectively called “Lys-plasmin” (11).

Recent experiments have also shown that the number of tPA-binding sites (12) and fibrinolytic rates for thin fibers (13) is dependent on fibrin structure. In this report, our aim was to manipulate fibrin fiber structure in an effort to examine the effect of fibrin fiber diameter and density on both the enzymatic action of plasmin on fibrin and the activation of plasminogen by tPA.

MATERIALS AND METHODS

Fibrinogen Purification—Human plasma was thawed in the presence of 25 KIU/ml aprotinin and 10 μ M D-phenylalanyl-N-[4-[(aminoiminomethyl)amino]-1-(chloroacetyl)butyl]-L-prolinamide 180(PPACK). Plasminogen and fibronectin were removed by a lysine and gelatin affinity columns respectively. Plasminogen- and fibronectin-free fibrinogen was then obtained by ammonium sulfate precipitation and DEAE ion-exchange chromatography. Coagulable protein was greater than 95%. Fibrinogen concentration was measured spectrophotometrically using an extinction coefficient of 1.51 mg/ml. Human α -thrombin was a gift from Dr. Frank Church (University of North Carolina). Highly purified plasminogen (9.6 casein units/ml) was purchased from Kabi Vitrum and two-chain tissue plasminogen activator (Duteplase, Burroughs Wellcome, Research Triangle Park, NC, 3.1×10^5 IU/ml) was a gift from Dr. Henry Burger.

Fibrin Gel Assembly—Fibrin gels were formed directly in 1-cm polystyrene cuvettes by mixing purified fibrinogen solutions (1 g/liter) with buffered solutions of α -thrombin. Unless otherwise stated, the final thrombin concentration was 1 NIH units/ml. Gels were formed at 0.05 M Tris, pH 7.4, 5 mM CaCl₂, and the ionic strength adjusted to 0.15 with NaCl. All measurements were made at 37 °C. Fibrinogen was added at time 0 to the buffered human α -thrombin solution containing specified additives and the turbidity (τ) followed as a function of time. The first appearance of the gel is indicated as an increase in turbidity. Fibrin structure was modified in two different ways. The first method was to alter the assembly rate by varying the ratio of thrombin to fibrinogen (14). In these experiments the fibrinogen concentration was held constant and the concentration of α -thrombin varied from 1 NIH unit/ml to 0.05 NIH unit/ml. A second method used a fibrin assembly inhibitor to alter fibrin structure (13). In these experiments fibrin structure was altered by addition of a radio contrast agent, iopamidol (Squibb), which induces formation of thin fibrin fibers, to the polymerizing solution. In all experiments, ionic strength and variation in polymerization buffers were carefully

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¹ The abbreviations used are: tPA, tissue plasminogen activator; KIU, kallikrein-inhibitor unit.

controlled so that the effect of changes in fibrin structure could be isolated and studied. All fibrinogen was quantitatively converted to fibrin regardless of the mass/length ratio.

Turbidity Measurements—Turbidity measurements were made at 37 °C with a Cary 118C spectrophotometer as previously described (14). The mass/length ratio for thin fibrin fibers, where the diameter of the fiber is less than the wavelength of the incident light, can be calculated from the wavelength dependence of turbidity (14) as given by:

$$\tau((88/15)\pi^3 n(dn/dc)^2 C \mu) / N \lambda^3 \quad (\text{Eq. 1})$$

τ is the sample turbidity, n is the refractive index, C is the concentration in grams/liter, μ is the mass/length ratio, N is Avogadro's number, and λ is the wavelength of the incident light. A plot of τ versus λ is linear, if the fibers are sufficiently thin, and permits calculation of the mass/length ratio. Refractive index measurements were made in a temperature-controlled Bausch and Lomb Abbe-3L refractometer. Refractive index increments were made in a Brice-Phoenix differential refractometer that was equipped with a HeNe laser as the light source.

Plasmin Assay—A two-stage assay was used to measure the plasmin concentration. Purified human plasminogen from Kabi Vitrum was reconstituted to 9.6 casein units/ml with water. In the first stage, fibrin was formed and plasminogen activated by tPA. To this end, fibrin gels were prepared by adding the prewarmed plasminogen solution (37 °) to a degassed solution of 0.05 M Tris, pH 7.4, 5 mM CaCl_2 , 1 NIH unit/ml human α -thrombin, 40 ng/ml tPA, and a variable amount of iopamidol. The fibrin assembly reaction was initiated by the addition of fibrinogen. The final concentration of fibrinogen was 1 g/liter and plasminogen was 0.008 casein units/ml. Ionic strength was adjusted to 0.15 with 1 M NaCl. The samples with negative and positive controls were incubated at 37 °C for 50 min after which time the reaction was stopped with hirudin (Sigma) and the fibrin clot removed. The second stage required addition of the plasmin synthetic substrate. After removal of the fibrin clot, an aliquot of the supernatant was taken and then diluted with a 80/20 volume ratio of 4.0 mM chromogenic substrate: S-2251 (CH-D-Val-Leu-Lys-pNA) from Kabi Vitrum. The concentration of plasmin generated by the conversion of plasminogen to plasmin by tPA for a given fiber size was obtained by comparison of the absorbance of the clot supernatant at 405 nm to the plasmin standard curve. A standard curve in the presence of fibrin for the hydrolysis of S-2251 by plasmin was obtained by serial dilution of Glu_1 -plasmin and determination of the absorbance at 405 nm. Independent assays using chromogenic substrates showed no effect of iopamidol on thrombin or plasmin activity.

RESULTS

In these experiments fibrin assembly and fibrinolysis is detected by changes in turbidity. The weight average mass/length ratio for the fibrin fibers is then calculated from the measured turbidity. The mass/length ratio is related to the fiber diameter and the fiber's mass density. Thus, the rate of fibrin assembly or fibrinolysis can be determined by a plot of the change in the fibrin fiber mass/length ratio as a function of time (14–20). The objective of these experiments is to show that fibrin structure as evaluated by mass/length ratio measurements influences the fibrinolytic rate.

Fig. 1 shows the sequence of fibrinogen activation by thrombin, the fibrin assembly resulting from the production of fibrin monomers, and fibrinolysis caused by tPA, plasminogen, and plasmin. Fibrin assembly is indicated by an increase in the mass/length ratio and fibrinolysis by a decrease in the mass/length ratio. Changes in the fibrin fiber structure in this figure are made by varying the ratio of thrombin to fibrinogen thereby altering the assembly rates and ultimately fibrin structure (14, 21). After addition of thrombin, the mass/length ratio increases indicating fibrin formation. Gel formation continues to grow over a period of 5–20 min as evidenced by the continued increase in the mass/length ratio. The initial increase in the mass/length ratio is followed by a decrease as fibrinolysis occurs. The negative slope of a plot of the mass/length ratio versus time estimates the fibrinolytic rate. Since

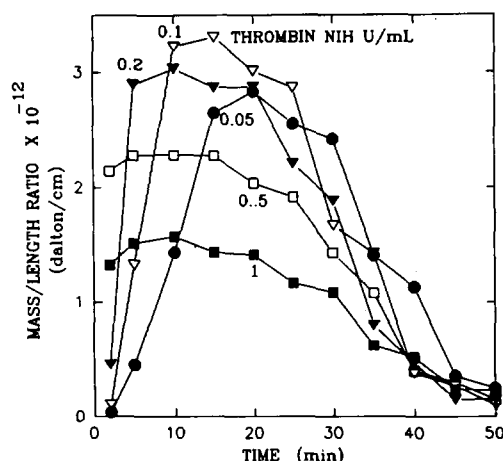


FIG. 1. The effect of fibrin fiber thickness on the fibrinolytic rate. The fiber diameter was changed by decreasing the thrombin concentration while holding the fibrinogen concentration (1 mg/ml) fixed. Thrombin concentrations (in NIH units/ml) were: 1 (■), 0.5 (□), 0.2 (▼), 0.1 (◇), and 0.05 (●). An increase in the mass/length ratio with time indicates fibrin assembly and a decrease in the mass/length ratio indicates fibrinolysis. As the ratio of thrombin to fibrinogen was decreased, the maximal fiber thickness increased (increased mass/length ratio). Also, as the thrombin concentration was decreased, the initial slope decreases as a consequence of slower assembly rates. The initiation of fibrinolysis (tPA 40 ng/ml, plasminogen 0.008 CU/ml) is indicated by a decrease in the mass/length ratio. The negative slope in this portion of the plot is a measure of the fibrinolytic rate. The fibrinolytic rate was slower for fibrin fibers with a low mass/length ratio (thin fibers). If the fibrinolytic rates were identical, the negative slopes would be parallel and would not converge.

initial slopes for fibrinolysis cannot be obtained from the plots because fibrin assembly may still be present during the early fibrinolytic period, estimates for fibrinolytic rates were obtained from a linear fit of the mass/length ratio data between the maximum (approximately 10 min) and minimum (approximately 40 min) values.

Three important features can be seen. First, as the thrombin concentration is decreased from 1 NIH unit/ml to 0.05 NIH unit/ml, both the gelation time and the rate of fibrin assembly is decreased. The slower assembly rate is shown by a flatter slope for the ascending portion of the curve (fibrin assembly) at lower thrombin concentrations. Second, as the concentration of thrombin decreases in the five separate experiments shown in Fig. 1, the maximum mass/length ratio increases indicating that thicker fibrin fibers are produced as the assembly process is slowed (21). Third, the fibrinolytic rate can be observed as a decrease in the mass/length ratio, which is seen in the terminal portion of the mass/length ratio versus time plots (22). The importance of this figure with respect to fibrinolysis is that the thicker fibers, which are formed at lower thrombin concentrations, have a more rapid decrease in the mass/length ratio per unit time than the thinner fibers, i.e. thicker fibers are lysed faster than thinner fibers. The steeper slope indicates a faster fibrinolytic rate. If the fibrinolytic rates for each different fiber thickness were the same, then a series of parallel curves with similar slopes would be seen.

A decrease in the fibrinolytic rate induced by thin fibrin fibers is also shown in Fig. 2. In Fig. 2 fibrin structure is changed by a different method. Here the addition of iopamidol causes the formation of thin fibrin fibers (23). In these experiments, the concentrations of thrombin and fibrinogen are held constant. As the fibers are made thinner by increasing the concentration of iopamidol (24) from 0 mM to 25.7 mM, flatter slopes and slower fibrinolytic rates are observed similar

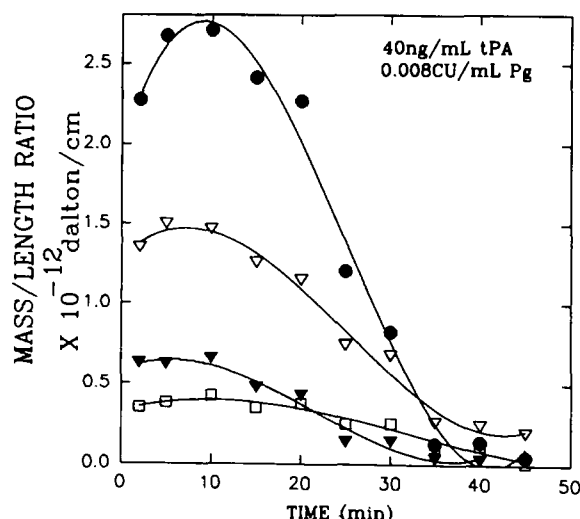


FIG. 2. The effect of fibrin fiber thickness on the fibrinolytic rate; thrombin, 1 NIH unit/ml, and fibrinogen, 1 mg/ml, were held constant. Thin fibers were induced by addition of iopamidol.² Iopamidol concentrations (mM) correspond to: none (●), 5 (◇), 10 (▼), and 29 (□). Fibrinolysis is indicated by a decrease in the mass/length ratio. The fibrinolytic rate decreased, i.e. a flatter slope, as the fibers were made thinner by iopamidol.

to those shown in Fig. 1. Thus, these experiments also suggest that the fibrinolytic rate is slower for thin fibers. In separate experiments no effect of iopamidol was observed on the hydrolysis of S-2251 by plasmin, indicating that iopamidol does not inhibit plasmin.

Since both the mass/length ratio for fibrin monomer and for fibrin fibers are known, the number of fibrin monomers contained within the fiber cross section can be calculated (14, 22). Further, the rate at which the fibrin monomers are removed from the fiber cross section during the fibrinolytic process can be determined from the time dependence of the decrease in the mass/length ratio. In Fig. 3 the rate of removal of fibrin monomers per minute from the fiber cross section is plotted against the number of fibrin monomers present within the fiber cross-section at the beginning of fibrinolysis. Data for this plot are obtained from Figs. 1 and 2. The slope of the plot is obtained by a linear regression and provides a simple rate constant for the process that is calculated to be $3.2 \times 10^{-2} \text{ min}^{-1}$. Thus, the fibrinolytic rate over the range of experimental conditions studied is linear with respect to the fibrin monomer density within the fiber.

Fig. 4 shows the effect of the fiber diameter on the conversion of plasminogen to plasmin by tPA with fibrin as a promoter. In these experiments fibrin structure is modified by iopamidol (23). Measurements were made at 37 °C. A two-stage assay was used. In the first stage fibrinogen is added to a buffered solution of α -thrombin, tPA, and plasminogen. Fibrin assembly followed by fibrinolysis is permitted to proceed as previously described. The action of thrombin is quenched by hirudin. In the second stage the clot is removed and the concentration of plasmin measured by hydrolysis of S-2251 at 37 °C. As the fibers are made thinner by increasing the concentration of iopamidol from 0 to 30 mM, the concentration of plasmin produced is decreased from 0.0085 to 0.0018 casein units/ml indicating the significance of fibrin fiber structure in the rate of conversion of plasminogen to plasmin.

In Figs. 1 and 2, fibrinolysis was dependent on the *in situ* production of plasmin by the action of tPA on plasminogen. A decrease in the fiber size was accompanied by a decreased rate of plasmin production. Experiments shown in Fig. 5 were

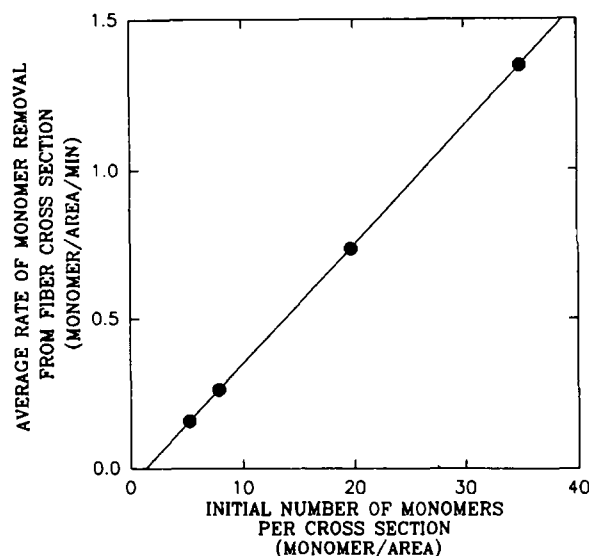


FIG. 3. The effect of the number of fibrin monomers within the fiber on the fibrinolytic rate was calculated from Fig. 2. The number of fibrin monomers within the fibrin fiber was calculated by dividing the fiber mass/length ratio by the fibrin monomer mass/length ratio. The initial number of fibrin monomers/fiber cross section was determined from the maximum mass/length ratio value. The time interval considered for the fibrinolytic rate calculation was chosen between the maximum mass/length ratio value and the break in the plot shown in Fig. 2, typically between 35 and 45 min.

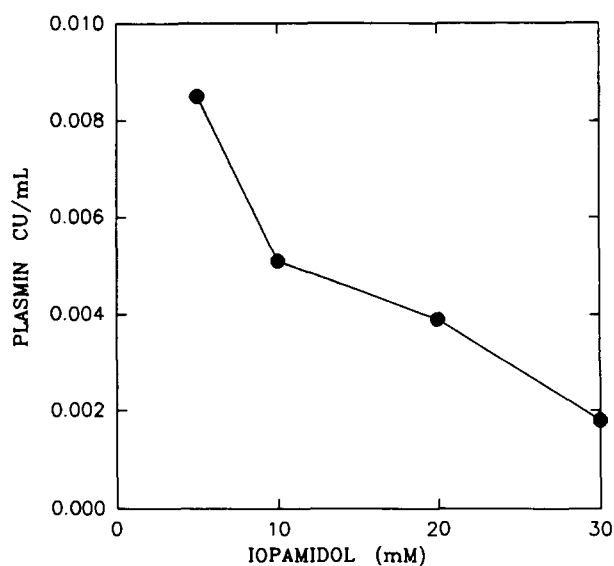


FIG. 4. The effect of fibrin fiber mass/length ratio on the conversion of plasminogen (0.008 casein units/ml) to plasmin by tPA (40 ng/ml). The fibrin fiber mass/length ratio is controlled by the concentration of iopamidol. As the fibers were made thinner, the amount of plasmin produced was decreased.

repeated under the same conditions with one exception: tPA and plasminogen were replaced by plasmin. This alteration of the procedure was designed to bypass the plasminogen activation step. No longer dependent on the generation of plasmin, thin fibrin fibers were shown to be a poorer substrate for plasmin than thick fibers. Fig. 5 demonstrates that in the presence of the same amount of plasmin, thin fibers have a slower fibrinolytic rate than thicker fibers. In fact, for fibers that are only a few monomers thick, i.e. a mass/length ratios of less than 0.5×10^{12} daltons/cm, a protective effect may be present. Hyper-rigidity, i.e. unusually stiff fibrin gels, has been observed early in fibrinolysis and may be related to this

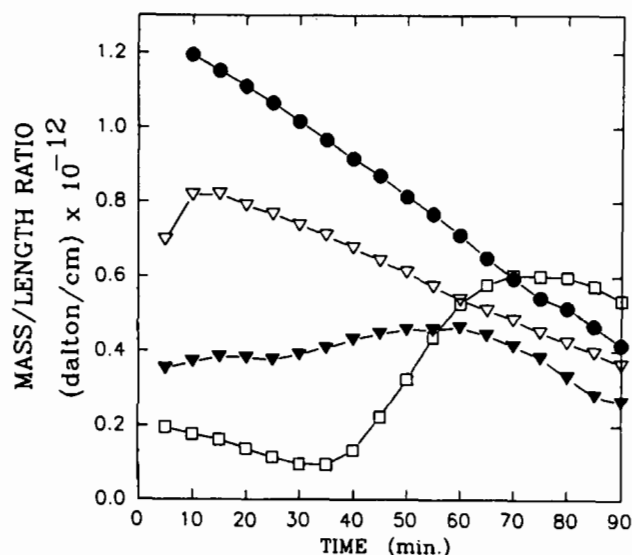


FIG. 5. Effect of fibrin fiber mass/length ratio on fibrinolysis initiated by plasmin. In these experiments plasmin 0.008 casein units/ml was substituted for tPA and plasminogen, so that plasminogen activation was not required for plasmin production as in Figs. 1 and 2. The fiber mass/length ratio was decreased by addition of iopamidol (mM) as follows: 0 (●), 5 (◇), 10 (▼), 20 (□). The fibrinolytic rate decreased as the mass/length ratio decreased until a critical mass/length ratio of 0.5×10^{12} daltons/cm was reached. No fibrinolysis occurred for fibers with a mass/length ratio less than this value until the fiber diameter grows larger to the apparent critical limit of approximately 0.5×10^{12} daltons/cm. 0.5×10^{12} daltons/cm corresponds to a fiber approximately 2 fibrin monomers thick.

protective effect (25). It is possible that fibrinogen, which is less susceptible to plasmin, may be incorporated into the gel during early gel formation.²

DISCUSSION

Fibrin structure varies, depending on the conditions under which it is assembled (14–21). The material properties, such as gel rigidity, fiber thickness, fiber mass density, pore size (the distance between the fibers within the gel), fiber branch point density, and stiffness of the fibers between the branch points are determined by the fibrin structure. Different physiologic or pathologic conditions may govern the structural features of fibrin. These features determine the mechanical rigidity of the gel (17, 24, 26), how easily other macromolecules can diffuse within the gel (27), and how long the gel may survive. The internal structure of fibrin influences the diffusion rates of macromolecules, such as plasminogen or tPA, within fibrin. For example, we have recently shown by holographic relaxation spectroscopy that the diffusion of aggregated IgG is influenced by pore size within fibrin (27), but that the diffusion coefficient for monomeric IgG and albumin is not.

Fibrinolysis is also influenced by fibrin structure. Fibrin structure has been implicated in determining the number of tPA-binding sites on fibrin (12) and resistance to plasmin attack (13). Ping and Gaffney (12) have observed that production of thick fibrin fibers is associated with adsorption of more tPA and faster fibrinolytic rates. Studies in this report show that plasmin production by tPA activation of plasminogen is proportional to the fibrin fiber thickness, which may provide greater surface for assembly of the fibrinolytic complex. As the concentration of the plasmin converted is in-

creased, the rate of fibrinolysis is expected to escalate. With thin fiber production, a decrease in the plasmin concentration and slower fibrinolytic rate is expected.

In addition to a decrease in tPA-binding sites on thin fibrin fibers, a decrease in plasmin-binding sites may also exist, as suggested by the occurrence of much slower fibrinolytic rates when plasmin is substituted for tPA and plasminogen as in Fig. 5.

Thus, at least two effects of the thin fibers on fibrinolysis are observed. One is a slower rate of plasmin production and the other is a slower rate of fibrin digestion by plasmin. The possible thread linking these two events may be derived from a decrease in binding sites for plasmin in thin fibers, or limited access to the binding sites by plasmin in thin fibers or a combination of both. Preliminary experiments using an enzyme-linked immunosorbent assay technique to detect the presence of plasmin bound to fibrin indicate that less plasmin is found on clots composed of thin fibers compared to clots formed from thick fibers.³ Ping and Gaffney (12) have found a decreased number of binding sites in gels formed under conditions that produce thin fibrin fibers. In this report, the rate of fibrinolysis with time is noted to change during the early time points, especially for the thick fibers, which also supports the observation that the fiber diameter influences the fibrinolytic rate.

It is possible to calculate the number of fibrin monomers present in the fibrin fiber cross-section using the mass/length ratio data. For example in Fig. 2 after 10 min of assembly time in the absence of iopamidol, the maximal fiber thickness occurs and is composed of an average of about 35 fibrin monomers in the fiber cross-section (Fig. 3). Beyond 10 min the fibrinolytic process begins to disassemble the fibers, and at 35 min the average fiber cross-section is about 2–3 fibrin monomers thick. Approximately 1.3 monomers/min are removed from the fiber cross-section in these fibers. In contrast, if the thin fibers are studied (induced by 20 mg/ml iopamidol) the starting number of fibrin monomers in the cross-section is an average of 5.25 and by 35 min is also reduced to 2–3 fibrin monomers in the fiber cross-section. For these thin fibers, approximately 0.09 monomer/min is removed from the fiber cross-section. Thus, the rates of monomer removal are approximately two orders of magnitude slower for the thin fibers compared to the thicker fiber. When the fiber cross-section is reduced to 4–5 fibrin monomers, a common fibrinolytic rate occurs.

Additional support for these experimental observations is derived from the decrease fibrinolysis of fibrin formed from the dysfibrinogen Chapel Hill III, which forms clots composed of extremely thin fibers. Fibrinolysis of the dysfibrinogen Chapel Hill III is normal, but fibrinolysis was drastically delayed (28). Another abnormal fibrinogen, fibrinogen Dusart, has a delay in lateral association with production of very thin fibrin fiber similar to fibrinogen Chapel Hill III and also has a decreased binding of plasminogen (29).

The basis for the slower rates is not completely understood at present. These data indicate that the fibrin structure at the beginning of the fibrinolytic process determines the kinetic rate; the greater mass/length ratio, the faster the fibrin dissolution. Since tPA and plasminogen binding may occur during fibrin assembly, the fiber structural features at the end of fibrin assembly may determine the number of potential binding sites for the fibrinolytic complex. It is probable that thin fibers possess fewer plasmin binding sites, which may be just a function of the decrease surface for the thin fibers. We postulate that thin fibers do not provide either an optimal

² J. McDonagh, unpublished data.

³ D. A. Gabriel, unpublished data.

surface for the tPA-plasminogen-fibrin complex binding or for plasmin binding. This phenomenon is possible due to 1) a decrease in the number of binding sites resulting from the smaller surface area of thin fibers or 2) steric factors caused by the increased curvature of the thin fibers that prohibit stable interaction between tPA and its binding site. This effect does not appear to be the result of changes in the diffusion coefficient for molecules diffusing within the fibrin gel, since photon correlation spectroscopy and holographic relaxation spectroscopic measurements of albumin and immunoglobulin G within the fibrin gel are not changed by similar modifications in fibrin (27, 30). Pulsed-field gradient nuclear magnetic resonance spectroscopy has been used to further validate these measurements in polyacrylamide gels (31). The radius of curvature in the thin fibers may be important since Nieuwenhuizen *et al.* (6) have shown a binding site for tPA on the γ -chain. Thus, it is possible that the spatial relation between the tPA-binding site on the γ -chain and the plasminogen-binding site on the α -chain may be critically distorted in thin fibers. Alternative explanations include a solution statistical factor of a decreased collision rate between plasmin and fibrin based on the fiber size since thin fibers make a smaller collisional target. Further, the composition of fibrin with respect to desA-, desAA-, or desAABB fibrin monomers or the occasional inclusion of a fibrinogen molecule may be important to tPA and plasmin binding. Thus, surface provided by fibrin for the fibrinolytic process is important as a means to regulate the fibrinolytic process.

In combination, these experiments show that the decreased fibrinolytic rate is not only a result of a slower production of plasmin for thin fibers, but also that thin fibers are more resistant to plasmin digestion than thick fibers.

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